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Published in:
Journal of Evolutionary Biology

DOI:
10.1046/j.1420-9101.2003.00510.x

Spatially structured genetic variation in a broadcast spawning bivalve: quantitative vs. molecular traits

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Keywords:

heterogeneous environments;
isolation;
Macoma balthica;
marine invertebrates;
Q_{ST};
selection.

Abstract

Understanding the origin, maintenance and significance of phenotypic variation is one of the central issues in evolutionary biology. An ongoing discussion focuses on the relative roles of isolation and selection as being at the heart of genetically based spatial variation. We address this issue in a representative of a taxon group in which isolation is unlikely: a marine broadcast spawning invertebrate. During the free-swimming larval phase, dispersal is potentially very large. For such taxa, small-scale population genetic structuring in neutral molecular markers tends to be limited, conform expectations. Small-scale differentiation of selective traits is expected to be hindered by the putatively high gene flow. We determined the geographical distribution of molecular markers and of variation in a shell shape measure, globosity, for the bivalve *Macoma balthica* (L.) in the western Dutch Wadden Sea and adjacent North Sea in three subsequent years, and found that shells of this clam are more globose in the Wadden Sea. By rearing clams in a common garden in the laboratory starting from the gamete phase, we show that the ecotypes are genetically different; heritability is estimated at 23%. The proportion of total genetic variation that is between sites is much larger for the morphological additive genetic variation ($Q_{ST} = 0.416$) than for allozyme ($F_{ST} = 0.000$–0.022) and mitochondrial DNA cytochrome-*c*-oxidase-1 sequence variation ($\Phi_{ST} = 0.017$). Divergent selection must be involved and intraspecific spatial genetic differentiation in marine broadcast spawners is apparently not constrained by low levels of isolation.

Introduction

The life cycle of many sedentary species in the marine environment contains a free-swimming larval phase. This short period early in life, in which huge displacements may take place, contrasts strongly with a sedentary (e.g. corals, clams, coraline algae) or semisedentary (e.g. polychaetes, snails) adult stage. Geographically structured intraspecific genetic variation is generally not expected for such large, high-dispersal, panmictic populations, as diversification is traditionally assumed to take place in isolation (Mayr, 1942, 1963, 1982). Indeed, intraspecific molecular variation is usually unstructured over large distances in such species (e.g. Borsa *et al.*, 1991; Koehn, 1991; Palumbi, 1996). However, some well-known exceptions to this rule exist (e.g. oysters: Reeb & Avise, 1990; Karl & Avise, 1992; giant clams: Benzie & Williams, 1997). Also, marine species diversity can be high in the absence of barriers to dispersal (Grassle & Maciolek, 1992) and sibling species are more often sympatric or parapatric than allopatric (Knowlton, 1993).

Like their terrestrial counterparts, individuals of a single species of marine invertebrate may inhabit very different habitats, e.g. either the periodically exposed intertidal or the adjacent subtidal, or either the outside of rocks or the sheltered cracks in between. Here we address

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the question how marine invertebrates with pelagic larvae deal (in a population genetical sense) with the variation of environments contained within their range. More specifically, we investigate whether local adaptations can occur despite the lack of obvious barriers to gene flow.

Diversification by locally differing selection regimes is traditionally assumed to be prevented already by low levels of gene flow (Slatkin, 1987). An extensive body of theoretical work, on the other hand, shows how selective variation can be maintained because of spatial heterogeneity, even if mating and dispersal are random across niches (for reviews see Felsenstein, 1976; Hedrick *et al*., 1976; Hedrick, 1986). Empirical studies in agreement with selection-based spatial diversity are also emerging. Recently, selection, rather than isolation, has been put forward as an important factor in generating and maintaining diversity in birds (little greenbul *Andropadus virens*: Smith *et al*., 1997; blue tit *Parus caeruleus*: Blondel *et al*., 1999) and a lizard (Schneider *et al*., 1999). All three studies contrast the population structuring of neutral genetic variation at the molecular level with that of selective morphological traits and find that, although the former does not, the latter shows marked local divergence. Although in these studies the genetic basis for the morphological structuring is not demonstrated, the observations lead the authors to conclude that selection is a more important factor than geographical isolation in biological diversification.

In high-dispersal taxa like marine broadcast spawners with pelagic larvae, however, spatially structured selective genetic diversity is less likely, as it is the combination of partial isolation and selection that makes divergence-with-gene-flow feasible theoretically (Slatkin, 1987; Rice & Hostert, 1993). Spatial phenotypic differentiation may also be the result of developmental plasticity, driving the same genotypes into different directions in alternative surroundings. Transplantation experiments with adult individuals showed that phenotypic plasticity accounted for at least some of the geographical patterns of shell shape in, for example, the marine mussel *Mytilus edulis* (Seed, 1968) and the marine (but low dispersal) snail *Littorina obtusata* (Trussell, 2000; Trussell & Smith, 2000).

A quantitative approach which can provide insight into the relative roles of isolation and selection, is a comparison of the degree of differentiation in polymorphic molecular markers vs. quantitative traits of which the genetic basis is estimated. Standardized and equivalent measures for such comparisons are F_{ST} and Q_{ST}, respectively (Spitze, 1993; Merilä & Crnokrak, 2001). The neutral expectation for Q_{ST} is the value of F_{ST} for neutral single-locus genes in the same populations (Felsenstein, 1986; Lande, 1992).

Here, we document a geographical, habitat-related pattern in shell shape of the broadcast-spawning bivalve *Macoma balthica* (L.): shells from the Dutch Wadden Sea are more globose than those from the adjacent North Sea. This pattern is contrasted with the distribution of neutral molecular marker variants. Using laboratory-reared offspring, we then test to what extent additive genetic effects contribute to the shell shape pattern we observed. The null hypothesis that differentiation in quantitative trait genetic variation is similar to that in molecular marker variation is evaluated by comparing F_{ST} and Q_{ST} values.

Materials and methods

Study populations

Macoma balthica (L.) is a tellinid bivalve that lives buried in muddy to sandy sediments in the shallow subtidal to high intertidal along the coasts of Europe and North America. Its pelagic larvae drift in the water column for approximately 2–5 weeks (Caddy, 1969; Drent, 2002) before settlement in high-intertidal 'nursery areas' (Beukema, 1993). There they remain until their first winter, when secondary migration with the use of a mucoid drifting thread brings them to the place where they will spend their sedentary adult life (Beukema & De Vlas, 1989; Beukema, 1993). The clams do not produce gonads before they are 1-year old (Lammens, 1967).

The Wadden Sea is a soft-sediment tidal system enclosed between the mainland of the Netherlands and a chain of barrier islands (Fig. 1). Every low-tide water is transported out of the basin into the adjacent North Sea, exposing the mudflats. *Macoma balthica* is found in intertidal and subtidal areas of the Wadden Sea, as well

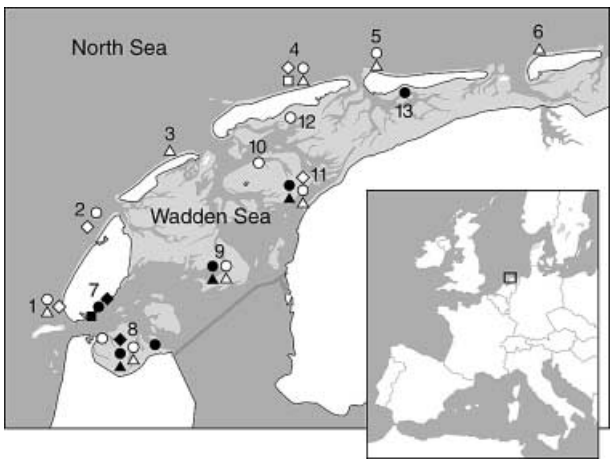

Fig. 1 *Macoma balthica* sampling sites. Intertidal areas are indicated with light shading. The sampling sites are numbered and circles indicate shell shape sampling sites, triangles allozyme sampling sites, diamonds mitochondrial DNA sampling sites, and squares quantitative genetics parental origins. The locations indicated on the map give approximate positions of the sampling sites. Open symbols represent subtidal sites, solid symbols intertidal sites.

as in relatively shallow subtidal areas of the North Sea outside the chain of islands. The habitats contained within this area differ in averages and fluctuations of water temperature (influenced by, e.g. wind and solar radiation) and salinity (depending on, e.g. rain, and mainland freshwater outlets), in sediment coarseness, water-current velocities, predation pressure and type (tidal regime rules the presence of, e.g. birds, predatory snails, crabs and fish), and in food type and abundance (see, e.g. Cadée & Hegeman, 1977).

The populations of *M. balthica* are discontinuous between the North Sea and the Wadden Sea. The tidal currents in the inlets between the islands are as high as 200 cm s^{-1} (Postma, 1957) and virtually no benthic invertebrates are found there (Van der Veer & Witte, 1993; own observations). The distance between populations of *M. balthica* on either side of the inlets is 2–4 km. The dispersal distance during the pelagic larval phase of *M. balthica* of 2–5 weeks can be roughly estimated as tens to hundreds of kilometres as follows: the turnover time of tidal basins in the Western Wadden Sea is 3–24 tidal cycles (Ridderinkhof *et al.*, 1990), i.e. <2 weeks. The dispersal potential is therefore certainly large enough to connect all the populations we study here in a single or a few generations.

To map shell shape variation in the field, in 1998, 1999 and 2000, we collected a total of 5342 individuals in 30 samples of *M. balthica* from 17 sites (Fig. 1). We *a priori* distinguished three different habitats: Wadden Sea intertidal, Wadden Sea subtidal and North Sea. Between two and five sites per habitat were sampled per year. (Note that for this field morphology survey as well as the allozyme survey, we distinguished these three habitats, whereas for the mitochondrial DNA survey and the common garden experiment, by which time we had become aware of a morphological difference between seas only, we focused on the two seas).

Common garden experiment

Ripe parents for the common garden experiment were collected in spring 1999 at one site in the Wadden Sea (Mokbaai) and one in the North Sea (Terschelling) (Fig. 1, sites 7 and 4, respectively) and were stored, buried in sediment, in aerated salt water containers below 10 °C in the laboratory. The clams were kept for 24 h in ultraviolet-irradiated, filtered (1 μm) seawater (UVFS) of ~0 °C prior to induction of spawning. They were then individually put into 120 mL glass beakers with 50 mL aerated UVFS of 15–18 °C containing Prozac® (Lilly, Windlesham, UK). Details of artificial induction of spawning are described in Honkoop *et al.* (1999). Sperm of a single male was added to eggs of a single female and the suspension left standing overnight at 15 °C. The eggs were then viewed under a binocular microscope at 60× magnification to check whether fertilization had been successful and development normal (i.e. embryos were spherical and several cleavages had taken place). During the following 3 days the embryos were kept in aerated perspex cylinders containing 2 L UVFS with 2.5×10^{-5} g L^{-1} streptomycin sulphate (Sigma, Zwijndrecht, the Netherlands) and 1.5×10^{-5} g L^{-1} penicillin (Merck, Haarlem, the Netherlands). The larvae were then transferred to aerated plastic bags containing 2 L UVFS containing the same concentrations of antibiotics as in the cylinder phase. The water of each batch was replaced every second day and an equal amount of algae (*Isochrysis galbana* and *Tetraselmis suecica* from continuous cultures) was added at the same time. After 24 days, when most larvae had developed a foot, which is a sign that a bivalve larva is competent to settle, they were transferred to 90 μm mesh carriers submerged in 400 mL UVFS to which the same concentrations of antibiotics and algae were added as in the bag stage. After 75 days of fertilization, when most larvae in a batch were larger than ~200 μm, the batch was transferred to an aquarium containing sand (maximum grain size <300 μm, heated for 5 h at 560 °C in order to remove organic material) and 1 L UVFS. In the aquarium phase the offspring were fed algae one to three times per week and the water was replaced once a week. The rearing temperature of the clams was kept constant at 15 °C.

In each of 10 separate spawning sessions that took place in May through August 1999, we obtained at least three male and three female spawners. Five spawning sessions were conducted using North Sea parents, and five with Wadden Sea parents. Of the parents, shell length and width (nomenclature according to Stanley, 1970) were measured to the nearest 0.01 mm using calipers. Parents were then frozen alive in liquid nitrogen and stored at −80 °C. Three males and three females were crossed in all possible combinations to produce nine fertilized batches. In this way we produced a total of 90 single-pair crosses. Upon reaching the aquarium phase, 76 batches still had at least one survivor. One year after spawning, shell length and width of offspring were measured to the nearest 0.01 mm using electronic calipers. If the offspring were smaller than ~5 mm in length, they were not measured in order to avoid the risk of damaging the shells. Of the 76 surviving batches, 72 contained offspring big enough to be measured. The total number of offspring thus measured was 919.

Molecular analysis

Live clams were frozen in liquid nitrogen to preserve enzyme activity and prevent DNA from degrading, and stored at −80 °C until further processing.

For allozyme analysis, a total of 345 adult Baltic clams was collected at 11 sites in spring 1997 (Fig. 1). A piece of tissue (avoiding the trematode parasites *Parvatrema* spp. present in a small percentage of animals in all populations) was ground in water using a porcelain mortar and cup and the lysate subjected to electrophoresis in 12% starch gels. Five enzyme loci were screened:

glucosephosphate isomerase (*Gpi*, EC 5.3.1.9), isocitrate dehydrogenase I and II (*Idh*, EC 1.1.1.43), phosphoglucomutase (*Pgm*, EC 5.4.2.2) and triosephosphate isomerase (*Tpi*, EC 5.3.1.1). All samples were run at 200 V for 8 h on a Tris citrate pH 7.0 buffer. Zymograms were visualized as described in Murphy *et al.* (1996) with some modifications. Gels always contained lysates of individuals from more than one site to enable unbiased scoring of alleles.

A 393 bp fragment of the mtDNA cytochrome-*c*-oxidase I gene was sequenced of a total of 138 adult *M. balthica* collected at six sites in spring 2000 (Fig. 1). DNA was extracted from approximately 1 mm^3 tissue using a standard phenol-chloroform extraction method and redissolved in 10 μL water. Phage Lock Gel Light (Eppendorf, Hamburg, Germany) during centrifugation at the phenol–chloroform stage enabled extraction in spite of the extreme mucoid nature of the tissue. If the individual was a ripe female, the gonad was used for DNA extraction, as this tissue contained relatively little mucus. In all other cases (ripe males and unripe or parasitized individuals) mantle tissue was used. Extracted DNA was visualized on 1% agarose gels. Polymerase chain reaction (PCR) amplifications were carried out in 50 μL volumes using a 1 : 10 dilution of DNA. In rare cases of failed amplification, either diluting the extract further to 1 : 100 or using the extract undiluted, usually rendered a positive PCR.

Universal primers LCO1490 and HCO2198 (Folmer *et al.*, 1994) were used to amplify a 710 bp fragment of the mitochondrial cytochrome-*c*-oxidase I gene. After preliminary sequence results were obtained, new primers internal to the universal primers were designed (forward: 5′-TTAGTGACTTCACACGGTTTGC-3′ and reverse: 5′-AGTACAGGTATAGCAACCACCAG-3′). The newly designed primers were used for the remainder of the study. Amplification products were cut out of 1% agarose gels and purified using the manufacturer's protocol (Qiaquick Gel Extraction Kit, Qiagen, Hilden, Germany). Single-stranded DNA was sequenced directly using the BigDye (PE Biosystems, Boston, MA, USA) sequencing kit and manufacturer's protocol with the forward primer on an automated sequencer (ABI PRISM 310 Genetic Analyser, Foster City, USA).

Statistical analysis

A shell shape measure was determined that can be described as globosity. Maximum shell length and shell width were measured. Shell shape displays positive allometry (larger shells are more globose), for which we corrected by taking the natural log of both shell measures and performing a principal component analysis (PCA) on the two measures [for a discussion of this method and a comparison with an analysis of covariance (ANCOVA) approach, see Sprent (1972)]. The first principal component is interpreted as size, and the second as size-corrected shape (globosity). In the remainder of this paper, for the term 'shell shape', read 'the factor loading of the second principal component of the PCA on log(shell length) and log(shell width)'. Separate PCA's were carried out with the data set for the description of shell shape variation in the field and that for the common garden experiment.

We fitted the corrected shell shape field data to the following mixed model:

$$z_{ijkl} = a + b_i + c_j + (bc)_{ij} + d_{ijk} + e_{ijkl}$$

where z_{ijkl} is an individual's shell shape, a is the population mean, b_i is the fixed effect of habitat i (Wadden Sea intertidal, Wadden Sea subtidal and North Sea), c_j is the random effect of year j $(bc)_{ij}$ is the interaction effect between habitat and year, d_{ijk} is the nested contribution for the k^{th} site within habitat i of year j, and e_{ijkl} is the error term for the l^{th} shell in site k of habitat i in year j. The expected mean squares of the model are specified in Table 1.

Shell shape data from the common garden experiment were corrected for length using PCA as described earlier. Heritability of shell shape was estimated using a regression of family average offspring shell shape [weighted for family size according to the method described by Kempthorne & Tandon (1953)] on midparent shell shape. This gives an estimate of the narrow-sense heritability, i.e. additive genetic variance as a fraction of total phenotypic variance. Matings were conducted within and not between origins, i.e. associatively, to maximize the power of estimating heritability (Reeve, 1961; Hill, 1970), within the constraint of a fixed number of families

Table 1 Mixed-model analysis of variance (ANOVA) of shell shape variation of *Macoma balthica* in the field.

Source of variation	d.f.	Expected mean squares	MS	*F*	*P*	s_x^2
a	$(i-1) = 2$	$\sigma^2 + l\sigma^2_{c\subset ab} + kl\sigma^2_{ab} + ikl\sigma^2_a$	172.6	22.1	<0.01	10.3%
b	$(j-1) = 2$	$\sigma^2 + l\sigma^2_{c\subset ab} + jkl\sigma^2_b$	2.56	0.18	n.s.	<1%
$a \times b$	$(i-1)(j-1) = 4$	$\sigma^2 + l\sigma^2_{c\subset ab} + kl\sigma^2_{ab}$	7.81	0.53	n.s.	<1%
$c\ (a \times b)$	$ij(k-1) = 25$	$\sigma^2 + l\sigma^2_{c\subset ab}$	13.68	18.49	<0.001	8.1%
Error	$ijk(l-1) = 5308$	σ^2	0.74			81.7%

Habitat *a* is treated as a fixed effect and year, *b* as a random effect; *c* are sites nested within year and habitat; habitats *i*, *j*, *k*, *l* are observed numbers associated with habitats, years, sites and individuals, resp.; s_x^2 are estimations of variance components σ_a^2, σ_b^2, σ_{ab}^2 and $\sigma_{c\subset ab}^2$, resp. Habitats recognized are Wadden Sea intertidal, Wadden Sea subtidal, and North Sea (always subtidal); n.s., not significant.

that we could rear. These analyses were carried out for the entire data set as well as for North Sea families and Wadden Sea families separately. We tested for maternal effects in a multiple regression containing shell shape of both mother and father as independent variables, a procedure adjusting from Falconer (1981, pp. 151–152).

A measure of quantitative genetic differentiation, Q_{ST}, was calculated from estimated within- and between-population additive genetic variances for shell shape. The narrow-sense heritabilities calculated as described above were used as estimators for the variance components, the within-population component being averaged over the two populations. Q_{ST} is calculated as $\sigma^2_{GB} / (\sigma^2_{GB} + 2\sigma^2_{GW})$, where σ^2_{GB} is the among-population component of variance for the quantitative trait and σ^2_{GW} is the within-population component of variance (Wright, 1951; Lande, 1992).

Allozyme data analyses were carried out using TFPGA 1.3 (Miller, 1997) and Arlequin 2.000 (Schneider *et al.*, 2000). Wright's *F*-statistics for a three-level sampling hierarchy were calculated using the method of Weir & Cockerham (1984) for sample size heterogeneity. Ninety-five percent confidence intervals for F_{IS} (fixation index within sites), F_{IT} (fixation index in total combined population), F_{ST} (proportionate reduction in heterozygosity of sites and regions relative to total combined population), and F_{RT} (proportionate reduction in heterozygosity of regions relative to total combined population) values were determined by bootstrapping over loci (1000 replications) and significance of individual *F*-statistics was determined by permuting the data 16 000 times: individuals among sites but within regions (F_{SR}; the proportionate reduction in heterozygosity of sites relative to that within regions), or sites among regions (F_{RT}) (cf. Excoffier *et al.*, 1992). Polymorphic loci were examined for Hardy–Weinberg equilibrium. Exact probabilities of conformity to Hardy–Weinberg equilibrium were approximated using a conventional Monte Carlo method.

DNA sequences were aligned by hand. A nested analysis of molecular variance (AMOVA; two populations, North Sea and Wadden Sea, with three subpopulations each) was carried out on the mtDNA sequence data, and a minimum spanning network among the haplotypes constructed, using ARLEQUIN 2.000 (Schneider *et al.*, 2000). Significance of the mtDNA Φ_{ST}, Φ_{SR} and Φ_{RT}, the haploid analogues of F_{ST}, F_{SR} and F_{RT} (Excoffier *et al.*, 1992), was calculated by permuting the data for 1000 replicates.

Results

Shell shape variation in the field

Shell shape variation is not randomly distributed over sites within the Wadden Sea and adjacent North Sea. Shells differ significantly between habitats, and also among sites nested within habitats and years (Table 1).

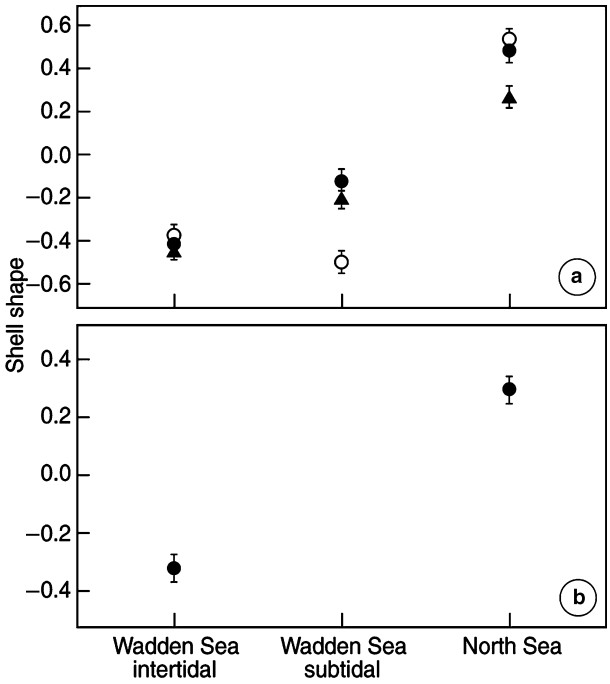

Fig. 2 (a) Mean shell shapes (±SE) of field collected *Macoma balthica* in three different habitats in three years. Triangles are data for 1998, solid circles for 1999, open circles for 2000. These data are least squares means from mixed-model analysis (Table 1). (b) Shape of offspring in common garden experiment: mean (±SE) for offspring of Wadden Sea ($n = 425$) and North Sea ($n = 496$) parents ($P < 0.001$, *t*-test).

The amount of variation explained by differences between habitats is comparable with that explained by differences among sites within habitats within years. Shape does not differ among years. The interaction between habitat and year is not significant, either (Table 1; Fig. 2a). The main difference among habitats is between the North Sea vs. the Wadden Sea (Fig. 2a). While the distributions of shell shapes in the Wadden Sea and North Sea display a considerable amount of overlap, Baltic clams are significantly more globose in the Wadden Sea [overall mean shell shape – 0.33 ± 0.015 standard error (SE), standard deviation (SD) 0.93, $n = 3658$] and in the North Sea their shells are flatter (mean shape 0.57 ± 0.020 SE, SD = 0.83, $n = 1684$; see Fig. 3).

Common garden experiment

Weighted parent–offspring regression for the entire data set shows that shell shape variation in the field is partly heritable (Fig. 4, Table 2). The slope of the regression, which indicates narrow-sense heritability, is estimated at 0.23 (standard error 0.080) and is significantly different from zero (weighted linear least squares regression, $P < 0.01$). The slopes of the parent–offspring regressions

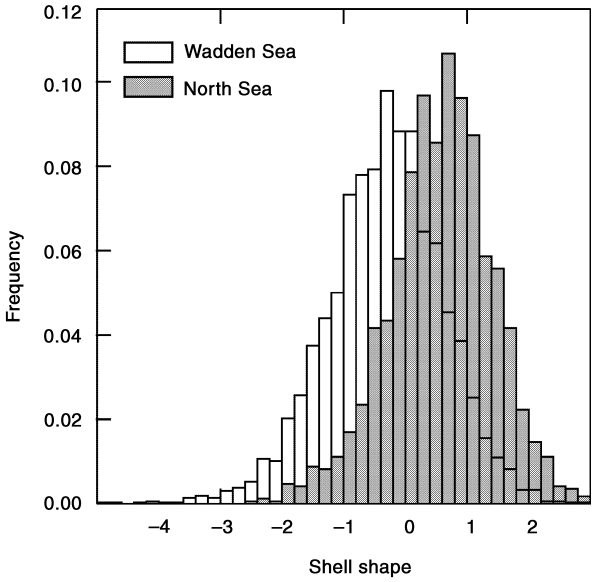

Fig. 3 Frequency distribution of shell shapes of *Macoma balthica* in the field for the western Dutch Wadden Sea (white bars) and the adjacent North Sea (grey bars). Data are lumped for 3 years (1998–2000) and sites within seas, $n_{total} = 5342$.

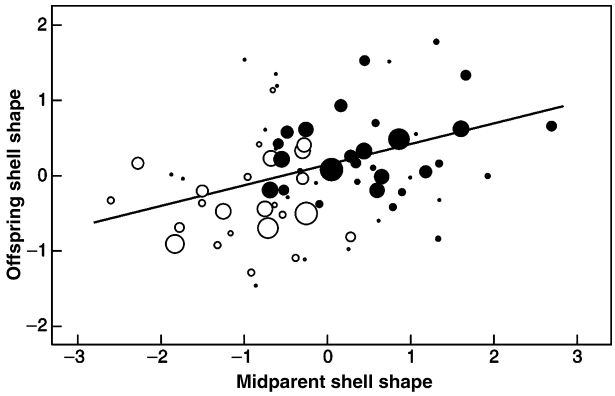

Fig. 4 Parent–offspring regression of mean offspring shell shape against midparent shell shape. Open symbols are Wadden Sea families, solid symbols North Sea families. Circle area corresponds linearly to family size; the larger the symbol, the larger the family. Value on axes is loading of second PCA factor; higher values indicate less globose shells. Line represents weighted least-squares regression ($y = 0.101 + 0.230*x$; $r^2 = 0.11$; $n = 72$).

for the two experiments separately are also positive (0.20 for the North Sea and 0.12 for the Wadden Sea, Table 2), but do not differ significantly from zero. There is no evidence for maternal effects, as the partial regression coefficient associated with paternal phenotype in the multiple regression ($0.5h^2 = 0.148 \pm 0.071$ SE) is not smaller than the maternal one ($0.5h^2 = 0.087 \pm 0.060$ SE) (b_2 and b_1, respectively, in Table 2). The proportion of quantitative additive genetic variance that is among populations is $Q_{ST} = 0.416$.

Molecular variance

The numbers of alleles detected per allozyme locus are 9, 3, 5, 9 and 5, for *Gpi*, *Idh1*, *Idh2*, *Pgm*, and *Tpi*, respectively. The locus *Idh1* is not considered polymorphic on the basis of the criterion that the frequency of the most common allele should not exceed 0.95; estimated overall frequency of its allele *b* is 0.98. Alleles are named *a*, *b*, etc. in order of electrophoretic mobility, such that *a* is the allele with the shortest electrophoresis distance.

Table 2 Weighted regressions from common garden experiment with *Macoma balthica*.

Data set	d.f.	MS	*F*	*P*	Parameter estimate (SE)
Midparent–offspring analyses					
All ($n = 72$ families)	1	5.106	7.966	0.006	$b = 0.227$ (0.080)
Error	70	0.641			
North Sea ($n = 38$)	1	0.877	1.541	n.s.	$b = 0.199$ (0.160)
Error	36	0.569			
Wadden Sea ($n = 34$)	1	0.223	0.384	n.s.	$b = 0.120$ (0.194)
Error	32	0.581			
Maternal effects analysis [All ($n = 72$)]					
Model	2	2.667	4.122	0.020	
Error	69	0.647			
	1	1.332	2.056	n.s.	$b_1 = 0.087$ (0.060)
	1	2.813	4.347	0.041	$b_2 = 0.148$ (0.071)

Weighting according to family size cf. Kempthorne & Tandon (1953). Parent–offspring analyses: fitted linear regression $y = c + b*x$. Maternal effects analysis: $y = c + b_1*x_m + b_2*x_f$; y = average offspring shell shape in a family; c = a constant; b = heritability h^2; b_1, b_2 = maternal resp. paternal components of heritability; x = midparent shell shape; x_1 = shell shape mother; x_2 = shell shape father; n.s. not significant.

Both permuting the data and bootstrapping over loci (Table 3) show that allele frequencies do not differ between habitats (model 1, $F_{RT} = 0.0015$, n.s.; nested analysis: Wadden Sea subtidal, Wadden Sea intertidal and North Sea, with three, three and five nested sites, respectively) or seas (model 2, $F_{RT} = 0.0030$, n.s.; nested analysis: North Sea vs. Wadden Sea with five and six nested sites, respectively). There is slight allozyme structuring among sites within regions (model 1, $F_{SR} = 0.0098$, $P < 0.05$; model 2, $F_{SR} = 0.0094$, $P < 0.05$). Pairwise site comparisons show that this structuring is present only within the Wadden Sea and that the difference is due to the Waardgronden (location 9 in Fig. 1) intertidal sample vs. the Waardgronden subtidal and the Balgzand (location 8 in Fig. 1) subtidal samples (permutation tests, $P = 0.018$ and $P = 0.009$, respectively). Inbreeding coefficients F_{IS} and F_{IT} are, however, high and different among loci (Table 4). For three of the four polymorphic loci, the shortage of heterozygotes is highly significant, also when taking into account multiple testing (exact test, $P < 0.01$ for *Gpi*, *Idh2* and *Pgm*). For *Tpi* there is a trend towards heterozygote deficiency. The proportion of total allozyme variation across loci that is between sites and habitats equals $F_{ST} = 0.0114$ (model 1), and between sites and seas (model 2) $F_{ST} = 0.0123$. Analysed per locus, F_{ST}s for model 1 are 0.007, 0.013, 0.009, 0.019, 0.001 and for model 2, 0.006, 0.015, 0.010, 0.022, 0.000 (for *Gpi*, *Idh1*, *Idh2*, *Pgm* and *Tpi*, respectively).

The number of mitochondrial COI haplotypes detected among the 138 individuals genotyped is seven (GenBank accession numbers AF443216 through AF443222). The haplotype set shows nine polymorphic sites, and the transition : transversion ratio is 8 : 1. Eight of the substitution polymorphisms are silent, i.e. do not correspond to an amino acid substitution, and one is a replacement substitution between alanine and valine ($k_a : k_s = 1 : 8$). The haplotype with the replacement polymorphism is haplotype *e* (Fig. 5, Table 5) and was found only once. Two haplotypes (*a* and *b*) are common and observed in

Table 3 Wright's *F*-statistics for five polymorphic allozyme loci. Data were examined using two hierarchical models (model 1: regions Wadden Sea subtidal, Wadden Sea intertidal and North Sea, with three, three and five nested sites, respectively; model 2: regions North Sea vs. Wadden Sea with five and six nested sites, respectively).

Model	F_{IS}	F_{IT}	F_{ST}	F_{RT}
1	0.137	0.147	0.0114	0.0015
	(0.070; 0.17)	(0.075; 0.19)	(0.0045; 0.018)	(–0.0062; 0.0049)
2	0.137	0.148	0.0123	0.0030
	(0.070; 0.17)	(0.076; 0.19)	(0.0045; 0.020)	(–0.0014; 0.0058)

Between brackets: 95% confidence intervals obtained from bootstrapping 1000 times over loci.

Table 4 Inbreeding coefficients F_{IS} and F_{IT} per allozyme locus; P = exact probability of conformity to Hardy–Weinberg equilibrium using conventional Monte Carlo method.

Locus	F_{IS}	F_{IT}	P
Gpi	0.117	0.122	0.000
Idh1	–0.027	–0.011	1.000
Idh2	0.112	0.122	0.002
Pgm	0.192	0.207	0.000
Tpi	0.050	0.051	0.093

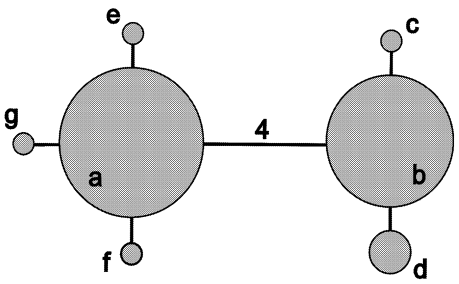

Fig. 5 Minimum spanning network for the seven detected mitochondrial haplotypes for 393 bp cytochrome-*c*-oxidase I fragment. Branch length is one substitution unless otherwise indicated. Bubble surface area indicates haplotype frequency across entire survey.

Table 5 Estimated mitochondrial DNA haplotype frequencies for 393 bp fragment of cytochrome-*c*-oxidase I gene in *Macoma balthica*.

		North Sea			Wadden Sea		
		Site 1	Site 2	Site 4	Site 7	Site 8	Site 11
Haplotype	GenBank accession no.	(22)	(24)	(25)	(25)	(21)	(21)
a	AF443216	0.55	0.42	0.52	0.60	0.38	0.67
b	AF443217	0.41	0.54	0.44	0.40	0.48	0.19
c	AF443218	–	–	0.04	–	–	–
d	AF443219	0.05	0.04	–	–	0.10	0.05
e	AF443220	–	–	–	–	–	0.05
f	AF443221	–	–	–	–	–	0.05
g	AF443222	–	–	–	–	0.05	–

Number of individuals per site in brackets; for location of sites see Fig. 1.

roughly equal frequencies. The other five haplotypes are rare. Figure 5 shows an unrooted minimum spanning network among these haplotypes. The two common haplotypes differ from each other by four substitutions. The rare haplotypes are one mutation away from one of the common ones. Estimated haplotype frequencies per sampling site are listed in Table 5. The populations are genetically homogeneous ($\Phi_{ST} = 0.017$, $\Phi_{SR} = 0.016$, $\Phi_{RT} = 0.0011$, in nested analysis of molecular variance, $n = 138$, n.s), indicating that there is neither a difference in haplotype frequencies between the North Sea and the Wadden Sea, nor between sites within seas.

Discussion

Molecular vs. quantitative markers of genetic population structure

Shells of *M. balthica* are more globose in the Wadden Sea than in the adjacent North Sea (Figs 2a and 3). The sites we sampled are very close to each other considering the potential scope for dispersal in this species; the North Sea and the Wadden Sea are separated by a chain of islands and the various connecting tidal channels are merely 2–4 km long, whereas the larvae remain for 2–5 weeks in the pelagic phase (Caddy, 1969). The two seas make up different environments with likely associated differential selection pressures, but the general expectation is that gene flow is high and will hinder selective differentiation.

The expectation that gene flow is ongoing at this level was tested and confirmed. Observed F_{ST}s, that range from 0.000 to 0.022 for allozymes and mitochondrial DNA sequences, correspond to a minimum of $M = 14$ migrants up to an infinite number of migrants per generation, assuming a classical island model of dispersal. This is much larger than the one migrant per generation which suffices (as a rule of thumb) to offset the diversifying effects of genetic drift (Slatkin, 1994). The hypothesis we thus formulate is that the two shell morphs in the two seas are the result of phenotypic plasticity. This hypothesis is rejected because variation in shell shape has a genetic component, and the offspring from one site in the North Sea and one in the Wadden Sea, reared in a common garden, still show the same distinct shell morphs as their parents (Fig. 2b). We can conclude that, despite ongoing gene flow, genetic differences among habitats do exist in this high-dispersal marine invertebrate species.

As a measure of spatial structure in additive genetic variance we estimated $Q_{ST} = 0.416$. The fact that Q_{ST} is much higher than the F_{ST} estimates means that the degree of differentiation in the quantitative genetic trait exceeds that achievable by genetic drift alone, and, consequently, that divergent natural selection favouring different phenotypes in different populations must have been involved to achieve this much differentiation (Whitlock, 1999; Merilä & Crnokrak, 2001). Recently, Q_{ST}/F_{ST} comparisons were reviewed by Merilä & Crnokrak (2001) and McKay & Latta (2002). When viewed in the context of these comparisons, it follows that the *M. balthica* data point is at the extreme end: high gene flow coupled with strong selection. Certainly, the *M. balthica* data emphasize the weak relationship between F_{ST} and Q_{ST}, which is interesting because of the general assumption of a link between neutral and non-neutral differentiation that is applied to many areas of biology.

Specifics of molecular and morphological data

Allozyme polymorphisms have been shown to be under balancing selection in oysters (Karl & Avise, 1992). Other types of selection on particular allozyme alleles have been demonstrated by several authors (e.g. Koehn *et al*., 1980; Johannesson *et al*., 1995). If, as suggested by Hummel *et al*. (1995, 1998), selection on allozyme polymorphism also plays a role in *M. balthica*, it is possible that our allozyme survey either overestimates or underestimates population subdivision. However, the F_{ST} estimated from the mitochondrial haplotype data, which are independent of the nuclear allozymes, is in the same order of the F_{ST}s from the allozyme data (unlike in the survey of Karl & Avise, 1992). The congruence between the nuclear and mitochondrial data sets does not support the idea that selection is an important determinant in spatial allozyme variation in this case.

Allozyme data with locus-specific heterozygote deficiencies form a pattern frequently found in bivalves (e.g. Lassen & Turano, 1978; Zouros *et al*., 1980; Skibinski *et al*., 1983; Foltz, 1986; Fairbrother & Beaumont, 1993). Proposed causes include Wahlund effects (sampling from populations with different allele frequencies), differential selection acting on larvae and/or adults, inbreeding, genomic imprinting, somatic aneuploidy and null alleles. Our allozyme data have been examined in more detail (P.C. Luttikhuizen, unpublished observations), showing that a technical artefact related to the large number of alleles causes the heterozygote deficiencies, while allele frequency estimates are essentially unaffected. The higher-level statistics F_{SR}, F_{RT} and F_{ST} therefore remain reliable indicators of the extent of allozyme population differentiation.

Both the amount of sequence variation and the absence of population subdivision at this spatial scale are comparable with other studies of mitochondrial DNA haplotypes of high-dispersal marine invertebrates (Brown & Paynter, 1991; Barber *et al*., 2000). The level of population subdivision here observed is also not larger than in comparable studies at larger geographical scales (Reeb & Avise, 1990; Small & Chapman, 1997; Wares *et al*., 2001). It is remarkable that the two most abundant haplotypes have a 1% sequence difference of four nucleotide substitutions between them. None of these differences amounts to an amino acid substitution at the

protein level, so natural selection cannot explain their coexistence. The two haplotypes are distributed evenly across seas (Table 5). An alternative scenario to a sympatric haplotype origin and subsequent lineage sorting in some populations but not the areas studied here, is that the two haplotypes arose in allopatry during an historic split, and that the two separate populations have since merged.

The heritabilities estimated from the separate analysis of common garden experiment data within the two origins Wadden Sea and North Sea are positive but do not significantly depart from zero. This can either mean that the power of these analyses is not large enough to detect an existing effect, or, alternatively, that there is no heritable variation within these populations. The positive heritability estimates from the separate analyses, together with the higher power of the between-origin experimental design (larger *n*, larger parental phenotypic range and mating performed associatively in order of increase power), argue in favour of the existence of some within-population heritable shell shape variation.

The common garden experiment shows that offspring from the Wadden Sea and the North Sea differ in shell shape from each other in the same way their parents do (Fig. 2a,b). The heritability of shell shape is estimated from parent–offspring regression at 23%. Phenotypic plasticity may therefore also contribute to population differences in the field. For our hypothesis, the crucial information from the experiment is that globosity possesses a significant heritable component, and that therefore the adult populations are genetically different.

Subtle barriers to gene flow and differential selection pressures

Intraspecific covariance between phenotypes and environmental heterogeneities can be due to phenotypic plasticity, to genetic effects, or (most often) to a combination of both. We have shown here that in *M. balthica,* genetic effects contribute to shell shape variation that is nonrandomly distributed over habitats. Models addressing the issue of how alternative genetic variants may inhabit different habitats within a species' range, when gene flow is ongoing, go back a long way. One of the first attempts to understand the problem was Levene (1953), who showed that under random mating, the distribution of fitness across niches must satisfy quite stringent conditions for stable polymorphism to occur. Stable equilibrium becomes more likely when, for example, constant dominance is replaced with reversible dominance, where the heterozygote is always closest in fitness to the fittest homozygote (Gillespie, 1976). The scope for stable maintenance of variation is also broadened by the introduction of habitat preference, i.e. a preference for niches associated with genotype (Hedrick, 1990; Jaenike & Holt, 1991), as well as reduced gene flow between niches (Brown & Pavlovic, 1992; Meszéna *et al.*, 1997). Local mating was originally suggested by Levene (1953) as a relaxing agent, but Strobeck (1974) has shown that in Levene's simple model, the parameter space for stable equilibrium is identical with or without local mating. However, in more complex models, e.g. one that already incorporates habitat preference, adding local mating does increase robustness (Hoekstra *et al.*, 1985). Polygenic models also generally show that reduced gene flow and drift are important facilitators of stable maintenance of polymorphism (e.g. Barton, 1986; Phillips, 1996).

Genetic drift is unlikely to play an important role in this case, as populations of *M. balthica* are very large. The system characteristics for understanding maintenance of geographically structured selective polymorphism here must be one or several of the following: (a) gene flow is reduced, (b) selection is extremely strong, (c) there is some form of habitat preference, or (d) mating is a local event.

There is some recent evidence that, contrary to expectation, marine larvae may be retained near source populations, constituting reduced gene flow (e.g. Jones *et al.*, 1999; Swearer *et al.*, 1999). The levels of gene flow might be high enough to prevent neutral genetic markers from differentiating, but low enough to aid population subdivision if selection plays a role. Interesting in this respect is the fact that the F_{ST} from allozymes for *M. balthica* is, although small, significantly different from zero, as assessed using bootstrapping over loci (Table 3). This may indicate that mixing is not complete, and that therefore a potential for reduced gene flow exists. In addition, neutral marker differentiation may, in this case, not yet have reached equilibrium between genetic drift and gene flow.

The strength of selection is potentially very large, as in *M. balthica* post-settlement densities decrease from up to 80 000 m^{-2} for newly settled recruits (Günther, 1999; Bouma *et al.*, 2001), to around 100 m^{-2} for adults (e.g. Honkoop *et al.*, 1998). Given our inference that the geographical pattern in morphology of *M. balthica* is maintained by selection in the face of gene flow, the question arises as to what the nature of the selective agents might be. The type of shell shape variation we investigate here has three main effects on mortality. First, being washed out of the sediment poses a great risk for burying bivalves (Yonge, 1950). In the more unstable sediments of the North Sea, the chances to become washed out are probably higher than in the finer sediments of the Wadden Sea, because of high current velocities and sand resuspension (Postma, 1957). Shell shape also affects predation risk. In the North Sea, we find relatively slow predators such as crabs and moon snails Naticidae (Holtmann *et al.*, 1996). It may be possible for burying bivalves to escape such slow predators by burying deeper or moving away. Flatter shells enable greater speed and range (Trueman *et al.*, 1966; Stanley, 1970; Vermeij, 1987; McLachlan *et al.*, 1995). Possible selection pressures may be the washing out or

selective predation of the more globose shells in the North Sea. Avian predators, that feed on *M. balthica* in the Wadden Sea intertidal, are much faster. Buried prey usually have size windows outside of which they are not profitable (Piersma *et al.*, 1993, 1994; Zwarts & Wanink, 1993). When the upper limit to ingestibility by an avian predator is set by the minimum diameter of a shell (Zwarts & Blomert, 1992), a more globose shell of the same size will be harder to swallow. Another hypothetical advantage of being globose in relation to avian predation is the larger amount of salt water trapped in the space between the valves. Ingestion of this excess of salt water makes it necessary to excrete the salt via the salt glands and this may be energetically very costly to the birds (Visser *et al.*, 2000).

Habitat preference at first glance does not seem feasible for drifting pelagic larvae, as the speed of currents is orders of magnitude larger than larval swimming speed. However, many traits have evolved that do enable pelagic larvae to have some control over where they settle, e.g. body density for buoyancy, mucus strands for increased drag, and delayed metamorphosis (for a review see Young, 1995). Experimental evidence also suggests that pelagic larvae actively select substrate type for settlement (Ahn *et al.*, 1993; Snelgrove *et al.*, 1999). In *M. balthica*, shell dimensions have a strong impact during secondary migration, which it undertakes with the use of a mucoid thread in its first winter, taking it away from its high intertidal nursery areas into the entire range of habitats they will inhabit as adults (Beukema, 1993). Beukema & De Vlas (1989) found that post-migratory Baltic clams that had settled in the North Sea are smaller as distance to the source (the Wadden Sea) increases. With experiments they demonstrated that this is because smaller shells sink more slowly. In addition to shell length, shell shape might also influence the time it takes for a migrating shell to sink. Baltic clams might thus be sorted nonrandomly with respect to shell shape over habitats during secondary migration.

The scale of mating in marine broadcast spawners is extremely small (metres; Pennington, 1985; Yund, 1990; Levitan, 1991; Babcock *et al.*, 1992; pers. obs.), relative to the scale of dispersal (up to tens or hundreds of kilometres). This is a consequence of the constraints imposed by the system of external fertilization (for a review see Levitan, 1995). Unless no selection whatsoever occurs, on account of the adults being sedentary, phenotypes will mate more often with similar phenotypes than under random mating.

Taking these considerations into account, the important characteristics of broadcast spawners for viewing them in the light of the maintenance of variation in heterogeneous environments are: global larval dispersal with the possibility of larval retention, larval habitat preference, local mating of the sedentary adults, and potential for strong selection early in life.

Genetic diversity of broadcast spawners

Our null hypothesis was that differentiation in quantitative trait genetic variation is not larger than that in molecular marker variation in *M. balthica*. Furthermore, because molecular differentiation was found to be very small, the homogenizing effects of inferred gene flow are expected to hinder selective differentiation. Following similar reasoning, short-distance (short relative to inferred gene flow levels) bivalve phenotypic patterns have been *a priori* assumed to be the result of phenotypic plasticity only (e.g. Herbinger *et al.*, 1998; Soares *et al.*, 1998). We have shown in this study that this is not the case for the shell shape variation associated with habitat of *M. balthica* in the Wadden Sea and adjacent North Sea, which does have a significant genetic component.

These observations are important both for evolutionary theory and in the context of nature conservation. First, these data imply that the potential for selective spatial differentiation, when isolation is very limited, is greater than usually appreciated. In the case of high-dispersal marine invertebrates this is probably because of their particular life cycle characteristics: global larval dispersal – possibility of larval retention – juvenile larval selection – local mating of the sedentary adults – potential for strong selection early in life. Secondly, for commercially exploited marine invertebrates, often particular refuge areas are left undisturbed, so-called 'Marine Protected Areas' (Hall, 1999), or 'No-Take Zones' (Horwood, 2000). If intraspecific diversity is geographically structured although molecular marker studies do not demonstrate it, then harvesting from particular (types of) areas may decrease intraspecific diversity. Also, if the maintenance of intraspecific polymorphism in high-dispersal marine invertebrates is a dynamic process in the sense of Levene (1953), changing the relative proportions of particular niches by selective harvesting may disturb the dynamic equilibrium, and thereby indirectly impoverish the genetic make-up of a species.

Acknowledgments

We thank Andy Ruhmaloi, Anneke Bol, Oscar Bos, Jan Hegeman and the crews of NIOZ research vessels 'Griend' and 'Navicula' for practical help, Jaap van der Meer for statistical guidance, Pim Edelaar for helpful discussions, Herman Hummel, Sophie Launey and Dennis Hedgecock for technical advice on rearing bivalve larvae, the 'molecular course' of Steve Palumbi and Andy Martin at Friday Harbor Laboratories in 1998 for kick-starting our DNA work and Pieter Honkoop, without whose pioneering breeding attempts of *M. balthica* this work would not have been possible. The manuscript has benefited greatly from extensive comments on earlier versions by several anonymous reviewers. Pieter, P.C.L and T.P. were all supported by a PIONIER-grant of the

Netherlands Organization for Scientific Research (NWO) to T.P. This is NIOZ publication 3600.

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Received 30 May 2002; revised 18 September 2002; accepted 11 November 2002